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Loaded Silk Fibroin Aerogel Production by Supercritical Gel Drying Process for Nanomedicine Applications

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Silk fibroin (SF) is a protein that can be used in biomedical field thanks to its biocompatibility, biodegradability and mechanical properties. SF is generally produced in form of aerogel by freeze drying; but, during this process, the delicate nanostructure of the gel can be destroyed, due to the surface tension action on the polymer matrix. The polymer organization at nanoscale (i.e., nanostructure) is preferably required for cells cultivation to ensure their adhesion, differentiation and migration inside the artificial support (i.e., scaffold).
In this study, SF aerogels at 10 and 15 % w/w were produced by Supercritical gel drying, potentially useful for nanomedicine applications. SF aerogels were loaded with Ascorbic acid (AA) at 5 % w/w with respect the protein; it was selected since it should favor cell growth and, for example in case of bone tissue engineering applications, for osteoblast differentiation. The obtained aerogels were characterized by scanning electron microscopy and porosity analyses. Moreover, release tests by a spectrophotometer UV/Vis to measure AA release kinetics from SF aerogels and mechanical tests to determine the Young modulus of the produced SF aerogels were also performed. Supercritical assisted process allowed to preserve SF aerogel morphology at nanoscale, thanks to the near zero surface tension of the supercritical mixture (CO_2 + organic solvent) at the selected operative conditions (i.e., 200 bar and 35 °C). Moreover, AA release rate can be controlled as well as mechanical properties of the produced aerogels, depending on SF concentration in the starting gel.

1. Introduction

Silk fibroin (SF) usually obtained from the cocoons of the silkworm *Bombyx mori* has been explored as a versatile protein for the formation of films, fibers, microspheres and porous scaffolds for several applications, mainly in biomedical field (de Moraes et al., 2014). Because of its excellent biological compatibility and mechanical properties, SF has been selected for osteoblast, hepatocyte and fibroblast cell support matrix and for ligament tissue engineering (Lv et al., 2006). Lv et al. (2006) prepared SF scaffolds for tissue engineering applications using a novel freeze drying/foaming technique; they obtained fibroin scaffolds characterized by a porosity and pore size of 85.8±4 % and 109±20 μm, respectively, from 12 % SF starting concentration; whereas, the porosity and pore size of SF scaffolds prepared from 8 % concentration were 96.9±3.6 % and 120±30 μm, respectively. Other authors studied and optimized the SF gelation mechanisms. Wang et al. (2008) reported a novel method to accelerate SF gelation process through ultrasonication. They observed that ultrasonication induced the formation of β-sheets in SF structure by alteration in hydrophobic hydration, thus accelerating the formation of physical cross-links responsible for SF gel stabilization. K^+ at physiological concentration and low pH promoted SF gelation, which was not observed in the presence of Ca^{2+}. Lu et al. (2011) studied the mechanisms of SF electrogelation. They observed changes in morphology and secondary structures before and after gel formation to clarify factors that control electrogelation. In particular, under an electric field, SF nanoparticles aggregated to form nano- or micro-spheres with sizes ranging from tens of nanometers to several micrometers on the positive electrodes because of screening of the negative surface charge, which could otherwise prevent intermolecular self-assembly of SF in neutral solution. Li et al. (2013) determined that $CaCl_2$-ethanol solution may represent the most appropriate method to prepare SF as biomaterials, especially as carriers for drug delivery.

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Another crucial aspect is the scaffold production. Scaffold should possess specific characteristics, such as a suitable 3-D structure, high porosity and interconnected pores to allow nutrients transport to cells, a nano-morphology for cells adhesion, guidance and differentiation on and inside the artificial support, and suitable mechanical properties in dependence of the tissue that it would regenerate (Cardea et al., 2014a; Baldino et al., 2015a). Generally, freeze drying, phase separation and electrospinning are the most used techniques to produce scaffolds; but, they have some limitations, in particular difficulty in obtaining a complete solvent elimination and the preservation of the scaffold morphology at nanoscale. These aspects are crucial for scaffold performance, in terms of citotoxicity and cells adhesion, guidance and migration and other aspects related to cells behavior (Baldino et al., 2015b).
Processes assisted by supercritical CO_2 (SC-CO_2) can overcome these limitations thanks to the near zero surface tension of the supercritical mixture CO_2+solvent and the very high diffusion coefficient of the supercritical system that allow a complete solvent elimination from the sample (Baldino et al., 2015c), preserving the structure at micro and at nanoscale (Campardelli et al., 2015). Supercritical phase separation (Duarte et al., 2010; De Marco et al., 2014; Cardea et al., 2014b) and supercritical gel drying (Baldino at al., 2015c; Campardelli et al., 2015) are among the most used processes to produce scaffolds for biomedical applications.
The aim of this work is to produce natural and biocompatible SF-based aerogels loaded with Ascorbic acid (AA) by supercritical gel drying process to be used as scaffolds for nanomedicine applications. The obtained aerogels were characterized by scanning electron microscopy, porosity analyses, release tests by a spectrophotometer UV/Vis to measure AA release kinetics from SF aerogels and also mechanical tests were performed to determine the Young modulus of the produced SF aerogels.

2. Materials and Methods

2.1 Materials

Silk fibroin (SF) was kindly purchased from Micro-macinazione (Molinazzo di Monteggio, CH - Switzerland); a scanning electron microscope image of untreated SF is showed in Figure 1. L-Ascorbic acid (AA), M_w 176.12, Calcium Chloride and Ethanol were bought from Sigma Aldrich. Carbon Dioxide (99 % purity) was bought from Morlando Group S.R.L. (Napoli - Italy). Distilled water was produced using a laboratory water distiller supplied by ISECO S.P.A. (St. Marcel, AO - Italy). All materials were used as received.

Figure 1: Untreated SF powder.

2.2 SF+AA aerogels preparation

SF solutions at 10 and 15 % w/w were prepared using as solvent a mixture of $CaCl_2/H_2O/CH_3CH_2OH$ (molar ratio 1/8/2). The solutions were stirred for 30 min at 200 rpm and 90 °C. Then, AA at 5 % w/w with respect SF was added to the solutions. SF+AA aerogels were produced by Supercritical gel drying, using a homemade laboratory plant (Baldino et al., 2015c). It consists of a 316 stainless steel cylindrical high-pressure vessel with an internal volume of 200 mL, equipped with a high pressure pump (mod. LDB1, Lewa, Germany) used to deliver SC-CO_2. Pressure in the vessel was measured by a test gauge (mod. MP1, OMET, Italy) and regulated using a micrometering valve (mod. 1335G4Y, Hoke, SC, USA). Temperature was regulated using PID controllers (mod. 305, Watlow, Italy). At the exit of the vessel, a rotameter (mod. D6, ASA, Italy) was used to measure CO_2 flow rate (see Figure 2).
The high pressure vessel was filled with SC-CO_2; when the desired operative conditions were reached (i.e., 200 bar and 35 °C) drying was performed with a SC-CO_2 flow rate of about 1 kg/h for 4 h. A depressurization time of about 40 min was used to bring back the system at atmospheric pressure.

Figure 2: Laboratory plant used to perform the experiments.

2.3 Analytical methods

Field Emission Scanning Electron Microscopy (FESEM) was performed on SF+AA aerogels previously cryo-fractured using liquid Nitrogen; then, they were sputter coated with Gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 160 s and analyzed using a FESEM (mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany) to determine the aerogels morphology and to measure the mean diameter of the nanofibers forming the structure.

Porosity measurements were performed on SF+AA aerogels using an Ultrapycnometer 1000 (Quantachrome instruments, Florida, USA).

AA release kinetics from SF aerogels were measured in continuous using a Varian (mod. Cary 50) UV/Vis spectrophotometer, reading the absorbance of the sample at 210 nm (that is the wavelength at which AA shows maximum absorption) at room temperature. SF+AA aerogel was immersed in a Phosphate Buffer Solution (PBS) at pH= 7.4, to simulate human body environment.

Ethanol residues were measured by a headspace (HS) sampler (model 7694E, Hewlett Packard, USA) coupled to a gas chromatograph (GC) interfaced with a flame ionization detector (GC-FID, model 6890 GC-SYSTEM, Hewlett Packard, USA). Solvent was separated using two fused-silica capillary columns connected in series by press-fit: the first column (model Carbomax EASYSEP, Stepbios, Italy) connected to the detector, 30 m length, 0.53 mm i.d., 1 µm film thickness and the second (model Cp Sil 5CB CHROMPACK, Stepbios, Italy) connected to the injector; 25 m length, 0.53 mm i.d., 5 µm film thickness. GC conditions were the ones described in the USP 467 Pharmacopea with some minor modifications: oven temperature from 45 °C to 210 °C for 15 min. The injector was maintained at 135 °C (split mode, ratio 4:1) and helium was used as the carrier gas (5 mL/min). Head space conditions were: equilibration time, 30 min at 95 °C; pressurization time, 0.15 min, and loop fill time, 0.15 min. Head space samples were prepared in 20 mL vials filled with internal standard DMI (3 mL) and 500 mg of NaCL and water (0.75 mL), in which samples were suspended.

Mechanical properties of the aerogels were measured using an INSTRON 4301 (Instron Int. Ltd, High Wycombe, UK). Rectangular samples with a length of 35 mm and a mean thickness of 1.5 mm were analyzed using a 100 N load cell, at 1.5 mm/min and 21 °C. All samples were immersed in water for about 20 h before the test. The Young modulus is defined as the initial linear portion of the stress-strain curve. Five specimens were tested for each sample.

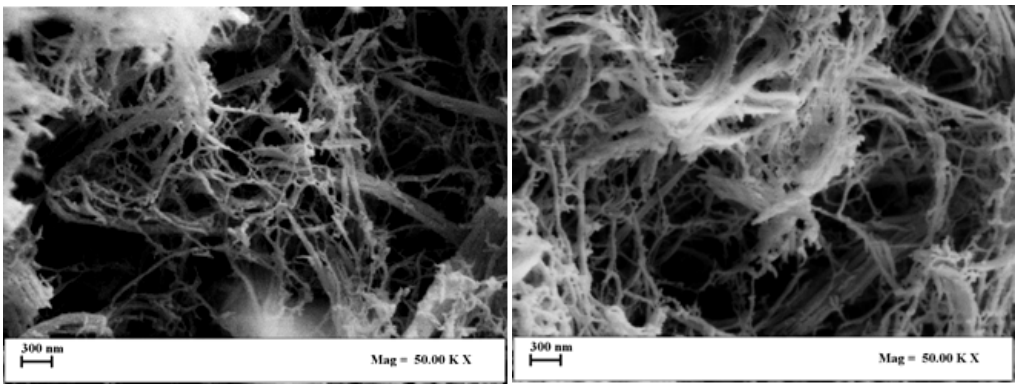
3. Results and Discussion

Aerogels are generally used in nanomedicine because their morphology at nanoscale is similar to natural tissue ECM (Campardelli et al., 2015). Nevertheless, the preservation of the native gel morphology is very

difficult during the drying process (e.g., freeze drying, air drying), since the surface tension exerted by the solvent on polymer matrix can lead to a structure collapse (García-González et al., 2011).
In Figure 3, a macroscopic view of a SF+AA aerogel is shown. It maintained the starting gel shape and volume after the supercritical process; this is the first evidence that the process did not affect the sample structure.

Figure 3: SF+AA aerogel picture.

Thus, SF+AA aerogel morphology was analyzed by FESEM. For each sample, AA amount was 5 % w/w with respect to SF.
In Figure 4, FESEM images of aerogels at 10 and 15 % w/w SF loaded with AA, processed by supercritical gel drying at 200 bar, 35 °C for 4 h, are reported.



(a) (b)

Figure 4: SF+AA aerogels processed at 200 bar, 35 °C for 4 h: (a) 10 % w/w SF; (b) 15 % w/w SF.

SF+AA aerogels were characterized by a nanofibrous morphology in both cases, with a mean fiber diameter of about 120 nm. However, SF+AA aerogel at 10 % w/w SF (Figure 4a) shows a more opened structure than SF+AA aerogel at 15 % w/w SF (Figure 4b), due to the lower SF amount in the starting gel, as expected. Porosity analyses showed values ranging between 88 and 93 % when the SF content decreases from 15 to 10 % w/w.
Therefore, the supercritical process has not destroyed the delicate nanostructure of the gel, since at the selected operative conditions, a supercritical mixture (CO_2 + solvent) was formed, allowing the drying process with a negligible surface tension. Moreover, from solvent residue analyses, we verified as the supercritical process also allowed a complete elimination of the used solvent (i.e., ethanol); indeed, ethanol values lower than 5 ppm have been measured.
Summarizing, as reported in the literature (Barnes et al., 2007), this kind of morphology is suitable for biomedical applications, since it favors cells adhesion, differentiation and migration on the biocompatible support.
In the second part of the work, AA release rate from SF aerogels was measured, to evaluate the release kinetics depending on SF gel concentration. In Figure 5, we reported the AA release curves on the same graph, that shows normalized AA concentration (i.e., AA concentration released at the time t, C_t, divided by the maximum AA concentration detected for that sample, C_{inf}) versus time.

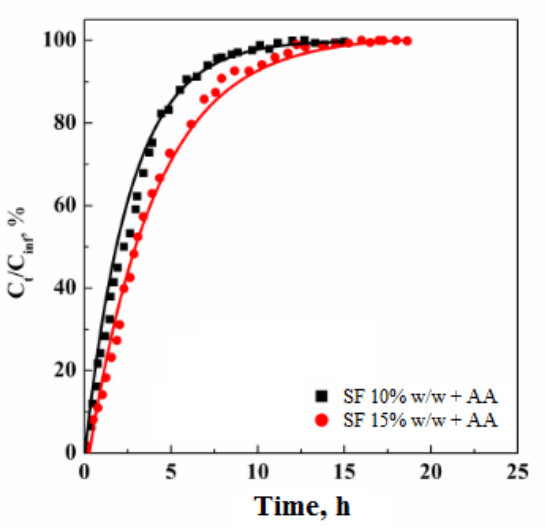


Figure 5: Comparison among AA release curves from SF aerogels at 10 and 15 % w/w, processed at 200 bar, 35 °C, 4 h.

In both cases, the release trend is exponential; but, the slope of the curves, thus, the release rate, changes. In particular, to reach the 50 % of the AA released, about 2.5 h and 2.8 h, for the SF aerogels at 10 and 15 % w/w are required, respectively. This is a consequence of SF aerogels morphology that influences the AA diffusion mechanism. In particular, when the aerogel structure is more opened due to the lower polymer concentration, the resistance to the mass transfer is lower, too; therefore, the AA release is faster if compared with the release from 15 % w/w SF aerogel. Operating in this manner, it is possible to control the release time in dependence of the application, by changing polymer content in the starting gel.
In the last part of the work, we focused our attention on the mechanical characteristics of the aerogels generated. Tensile mechanical properties were measured and the obtained results are reported in Table 1.

Table 1: Mechanical characteristics of the SF+AA aerogels produced by Supercritical gel drying

Aerogel	Young modulus [MPa]	Tensile Strength at break [MPa]
10 % w/w SF + AA	0.97	1.46
15 % w/w SF + AA	0.81	1.99

The results are very interesting because show a clear effect of the SF starting concentration on the mechanical properties of the aerogels produced. Indeed, as expected, the aerogel characterized by a lower SF content (i.e., 10 % w/w) presents an higher Young modulus (0.97 MPa) and a lower tensile strength at break (1.46 MPa). On the contrary, when the SF concentration is higher (15 % w/w), the structure is less elastic (lower Young modulus, 0.81 MPa), but strongest (Tensile strength at break 1.99 MPa).

4. Conclusions

In this work, biocompatible SF aerogels loaded with AA are successfully prepared by supercritical gel drying. The obtained nanofibrous morphology as well as porosity values make these aerogels suitable for nanomedicine application. Morphological characteristics and SF concentration influenced AA release kinetics and mechanical properties of the produced aerogels.

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